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# Myocardial β-Catenin-BMP2 signaling promotes mesenchymal cell proliferation during endocardial cushion formation

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## Abstract

Abnormal endocardial cushion formation is a major cause of congenital heart valve disease, which is a common birth defect with significant morbidity and mortality. Although β-catenin and BMP2 are two well-known regulators of endocardial cushion formation, their interaction in this process is largely unknown. Here, we report that deletion of β-catenin in myocardium results in formation of hypoplastic endocardial cushions accompanying a decrease of mesenchymal cell proliferation. Loss of β-catenin reduced *Bmp2* expression in myocardium and SMAD signaling in cushion mesenchyme. Exogenous BMP2 recombinant proteins fully rescued the proliferation defect of mesenchymal cells in cultured heart explants from myocardial β-catenin knockout embryos. Using a canonical WNT signaling reporter mouse line, we showed that cushion myocardium exhibited high WNT/β-catenin activities during endocardial cushion growth. Selective disruption of the signaling function of β-catenin resulted in a cushion growth defect similar to that caused by the complete loss of β-catenin. Together, these observations demonstrate that myocardial β-catenin

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Conflict of interest

There is no conflict of interest to be disclosed.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.yjmcc.2018.09.001.

signaling function promotes mesenchymal cell proliferation and endocardial cushion expansion through inducing BMP signaling.

**Keywords**

Endocardial cushion formation; Congenital heart valve disease; β-Catenin; BMP

## 1. Introduction

Heart valves control the unidirectional blood flow in the heart and are essential for normal cardiac function [1–4]. In mice, heart valve development begins with endocardial to mesenchymal transformation (EMT) to generate endocardial cushions within atrioventricular canal (AVC) and outflow tract (OFT) between embryonic (E) 9.5 and E10.5 [2,3,5–7]. From E11.5, these cellularized cushions undergo complex remodeling by proliferation and apoptosis of mesenchymal cells, as well as by deposition and organization of extracellular matrix, to form thin heart valve leaflets at birth [8–11]. Formation of endocardial cushions by EMT and remodeling is tightly regulated by regional molecular signals among endocardium, endocardium-derived mesenchyme, and myocardium [12]. Dysregulation of these signals can cause congenital heart valve defects, which are the most common congenital heart disease [12–16]. Therefore, characterization of the molecular signals among the valve-forming cells underlying the development of endocardial cushions will provide a better understanding of the etiology of congenital heart valve defects and help develop potential early disease diagnosis and interventions.

β-catenin is encoded by the *Ctnnb1* gene and has two distinct functions: maintaining cell-cell adhesion and mediating the canonical WNT (WNT/β-catenin) signaling [17]. WNT/β-catenin signaling plays critical roles in endocardial cushion formation. Key WNT pathway genes are expressed in the endocardium, mesenchyme, and myocardium of developing heart valves [8,18–20]. Multiple transgenic mouse models (TOPGAL, BATGAL, and $Axin2^{LacZ}$) that report WNT/β-catenin signaling indicate high nuclear activities of β-catenin in the endocardial cushion regions during heart valve development [20–22]. In particular, WNT/β-catenin signaling in endocardial lineage promotes EMT and post-EMT cell proliferation during endocardial cushion formation in zebrafish [23], chicken [24], and mouse [20,21]. In contrast to the intensive studies on WNT/β-catenin signaling in endocardial-lineage, the roles of myocardial WNT/β-catenin signaling in endocardial cushion development is less defined.

BMP signaling is another key regulator of endocardial cushion development. Multiple BMP ligands and receptors are expressed in cushion region during heart valve development [25]. *Bmp2* and *Bmp4* are expressed in AVC and OFT myocardium, respectively and required for EMT and post-EMT cushion growth [26–30]. Recently, BMP2 in endocardial lineage has also been reported to regulate AVC cushion maturation [31]. Interactions between WNT/β-catenin and BMP signaling have been reported to regulate early events of cardiogenesis including the formation of first and second heart field, as well as the looping morphogenesis of the heart tube [32]. *Bmp4* is a downstream target of WNT/β-catenin signaling in second

heart field [32]. Our previous *ex vivo* studies have suggested an endocardial–WNT to myocardial-BMP signaling axis underlying EMT process [33].

In this study, by using a myocardial-specific β-catenin knockout mouse model, we provide the genetic evidence to support that *Bmp2* is a mediator of WNT/β-catenin signaling in AVC cushion myocardium to promote mesenchymal cell proliferation and cushion growth in a paracrine manner.

## 2. Methods

### 2.1. Experimental mouse models

All mouse experiments were performed according to the guideline of the National Institute of Health and the protocol approved by the Institutional Animal Care and Use Committee of Albert Einstein College of Medicine. Noontime on the day of detecting vaginal plugs was designated as E0.5. Adult mice and mouse embryos were PCR genotyped using tail and yolk sac DNA, respectively.

### 2.2. Histology and immunofluorescence staining

Embryos were dissected between E9.5 and E15.5, and processed for paraffin sections as described previously [8]. Hematoxylin and eosin (H &E) and immunofluorescence staining were performed using standard protocol. The stained tissue sections were examined and photographed using a Zeiss Axio Observer Z1 and a Leica confocal microscope.

### 2.3. EdU and TUNEL assays

Cell proliferation and apoptosis were detected by using EdU and TUNEL assays respectively. Pregnant female mice were injected with EdU (Life Technology) through intraperitoneal injection at a concentration of 100 mg/kg. After a 2-h pulse, embryos were collected and processed for frozen sections. Serial sections crossing the entire cushion region were first stained with PECAM1 antibody followed by EdU staining with EdU imaging Kit (Life Technology) and counterstained with DAPI (Vector lab). The stained sections were photographed using a confocal microscope. The TUNEL assays were performed according to the manufacture's instruction.

### 2.4. RNA extraction and quantitative PCR (qPCR)

Total RNAs were isolated from pooled heart tissues using Trizol (Invitrogen). First strand cDNA was synthesized using the Superscript II Reverse Transcriptase Kit (Invitrogen). qPCR was performed using the Power SYBR Green PCR Master Mix (ABI). Gene specific primers were used (Table S1).

### 2.5. RNA in *situ* hybridization

RNA *in situ* hybridization (ISH) was performed as previously described [33]. Digoxigenin-labeled complementary RNA probes for *Bmp2*, *Msx1*, and *Msx2* were prepared from the linearized plasmids by reverse transcription. The stained embryos were then post-fixed, dehydrated, embedded in paraffin, sectioned and photographed using the Zeiss Axio Observer Z1 inverted microscope.

### 2.6. Cell proliferation assays on heart explants

AVC tissues were microdissected out from E10.5 control and β-cat$^{mKO}$ embryos and cultured on the rat-tail collagen gel in 4-well plates. Explants were cultured in media with or without BMP2 (R&D systems, 200 ng/ml) for 48 h. The cell proliferation was detected using EdU assays as described above.

### 2.7. Statistical analysis

Statistical analysis was performed using Microsoft Excel. All data were presented as mean ± SD. Student's *t*-Test was used for comparison between groups. Probability (*p*) values < 0.05 were considered as significant.

Full methods are available at Supplemental Methods.

## 3. Results

### 3.1. Paracrine regulation of endocardial cushion growth by myocardial β-catenin

To determine the role of myocardial β-catenin in heart valve development, we generated myocardial-specific *β-catenin* knockout mice (β-cat$^{mKO}$, hereafter) by crossing floxed *Ctnnb1* (*β-catenin*) mice with *Tnt$^{Cre}$* mice. Lineage tracing the Tnt$^{Cre}$-marked cells using the Rosa26 lacZ reporter line showed that the Cre-mediated lacZ expression was high in AVC cushion myocardium and low in OFT myocardium, especially in the distal OFT myocardium (Fig. S1A). Immunostaining showed that β-catenin was expressed in the endocardium, mesenchyme and myocardium during endocardium cushion formation from E9.5 to E11.5 (Fig. S1B-D). The β-cat$^{mKO}$ embryos started to show decreased β-catenin protein level in the myocardium of AVC and proximal OFT cushions when compared to controls at E9.5 (Fig. S1B), consistent with the low level of Cre-mediated recombination at the distal OFT myocardium (Fig. S1A). By E11.5, β-cat$^{mKO}$ embryos had completely lost βcatenin protein in the myocardium of AVC and proximal OFT, while residual β-catenin protein remained in the myocardium of distal OFT (Fig. S1C and D). As expected, β-catenin expression in the endocardium or cushion mesenchyme was not affected in the β-cat$^{mKO}$ embryos (Fig. S1B-D). Disruption of myocardial *β*-catenin resulted in embryonic death before E14.5 (Fig. S1E). While gross morphology of E11.5 β-cat$^{mKO}$ and control (*β-catenin*$^{f/+}$ or *β-catenin*$^{f/f}$) embryos was indistinguishable, E12.5 β-cat$^{mKO}$ embryos were underdeveloped compared to their littermate controls and all E14.5 β-cat$^{mKO}$ embryos were dead (Fig. S1F and G). These results indicate that myocardial β-catenin is indispensible for embryo survival.

We then studied cardiac morphology by Hematoxylin and Eosin (HE) staining and found that E9.5 β-cat$^{mKO}$ embryos had comparable AVC and OFT cushions as their littermate controls (Fig. S2), indicating proper EMT had occurred. However, β-cat$^{mKO}$ embryos began to develop hypocellular AVC and OFT cushions at E10.5, despite having a normal cushion size (Fig. 1A, Fig. S3A and Fig. S4). By E11.5, β-cat$^{mKO}$ embryos exhibited smaller AVC and OFT cushions compared to controls (Fig. 1A, Fig. S3A and Fig. S5), suggesting that myocardial β-catenin is required for post-EMT cushion growth. Indeed, EdU assays revealed that β-cat$^{mKO}$ embryos exhibited decreased proliferation of endocardial, mesenchymal and

myocardial cells within the AVC and OFT regions (Fig. 1B and Fig. S3B). In addition, TUNEL assays showed that β-cat$^{mKO}$ embryos had significantly increased apoptosis of cushion mesenchymal cells (Fig. 1C and Fig. S3C). In contrast to the changes in cell fates, alcian blue staining revealed no difference in staining intensity or area in the AVC and OFT cushions between E10.5 control and β-cat$^{mKO}$ embryos (Fig. S6). Together, these findings demonstrate a critical paracrine function of myocardial β-catenin in regulating the proliferation and apoptosis of mesenchymal cells for the post-EMT growth of endocardial cushions.

### 3.2. Myocardial β-catenin regulates endocardial cushion growth through BMP signaling

To identify the factors that could mediate myocardial β-catenin to regulate the cushion growth, we performed quantitative PCR (qPCR) to examine the expression of candidate genes in AVC cushions of E9.5, E10.5 and E11.5 β-cat$^{mKO}$ embryos. Consistent with immunostaining, mRNA level of *β-catenin* was significantly reduced in β-cat$^{mKO}$ embryos at all three stages (Fig. S7A). The expression of *Tbx2* and *Tbx3*, transcriptional factors involved in AVC myocardium development, was slightly decreased at E9.5 and significantly reduced at E10.5 in *β*-cat$^{mKO}$ embryos. The expression of *Ccnd1* (a known target gene of *β-catenin*), *Ve-cadherin* (a gene involved in cell-cell adhesion that prevents EMT) and *Notch1* (a gene essential for EMT and post-EMT cushion growth) was not affected by loss of myocardial *β-catenin* (Fig. S7A). The expression of *Snail* or *Slug* (which encode transcription factors involved in EMT) was not affected at E9.5 and E10.5, but reduced at E11.5 (Fig. S7A). The expression of *Msx1* and *Msx2*, transcriptional factors essential for EMT, were differentially affected. While the expression of *Msx1* was not changed in AVC of E9.5 β-cat$^{mKO}$ embryos, and was slightly decreased in E10.5 and E11.5 β-cat$^{mKO}$ embryos, the expression of *Msx2* was dramatically diminished at all three stages (Fig. S7A). RNA *in situ* hybridization showed that the expression of *Msx2* in the cushion myocardium, but not in mesenchyme, was decreased in E10.5 β-cat$^{mKO}$ embryos (Fig. S7B). In contrast, *Msx1* was expressed predominantly in cushion mesenchyme at a comparable level between E10.5 control and β-cat$^{mKO}$ embryos (Fig. S7B). The expression of matrix genes (*Acan*, *Vcan*, *Has2*, and *Col1a1*) was not affected in AVC of β-cat$^{mKO}$ embryos, except that Acan was upregulated at E9.5. Although the impact of the change of *Acan* on endocardial cushion formation was not clear, the overall expression of matrix genes were comparable at E10.5 between control and β-cat$^{mKO}$ embryos.

Among the BMP pathway genes examined, *Bmp2* and *Tgfβ2* were significantly downregulated in AVC cushions of β-cat$^{mKO}$ embryos at all three stages (Fig. 2A and Fig. S7A). Consistently, RNA ,*in situ* hybridization revealed that *Bmp2* was highly expressed in the AVC myocardium of E10.5 and E11.5 control embryos, and such expression was markedly reduced in β-cat$^{mKO}$ embryos (Fig. 2B). Importantly, immunostaining revealed significantly reduced levels of phosphorylatedSMAD1/5/9 (pSMAD1/5/9) in the AVC cushions of E10.5 and E11.5 β-cat$^{mKO}$ embryos compared to controls (Fig. 2C and D), indicating reduced BMP signaling in the AVC cushion of β-cat$^{mKO}$ embryos and suggesting that BMP signaling may mediate the positive effect of myocardial *β-catenin* on AVC cushion growth.

We therefore performed rescue experiment to determine whether BMP2 mediates myocardial β-catenin-dependent mesenchymal cell proliferation. We cultured AVC tissues of E10.5 control and β-cat$^{mKO}$ embryos on collagen gel using media with or without recombinant BMP2 proteins. Of note, at this stage EMT is complete within the AVC cushion, allowing us to examine the post-EMT proliferation of mesenchymal cells. In this *ex vivo* system, the proliferation of mesenchymal cells is independent on the working function of cardiac chamber myocardium. Consistent with the *in vivo* findings (Fig. 1B and Fig. S3B), EdU labeling of explants showed that mesenchymal cells from β-cat$^{mKO}$ embryos exhibited significantly reduced proliferation and this reduction was fully rescued by adding BMP2 (Fig. 3A and B). These findings demonstrate that β-catenin in myocardium promote AVC cushion growth through inducing myocardial BMP2 expression and BMP/SMAD signaling in the cushion mesenchymal cells.

In contrast to AVC cushions, the OFT cushions of β-cat$^{mKO}$ embryos showed unchanged or slightly increased expression of BMP/Tgfβ ligands including *Bmp2*, *Bmp4*, *Bmp5*, *Bmp6*, *Bmp7*, *Tgfβ1*, *Tgfβ2*, and *Tgfβ3* (Fig. S8). The β-cat$^{mKO}$ embryos showed increased expression of mesenchymal cell markers (*Snail* and *Slug*) and the matrix genes (*Acan*, *Vcan* and *Has2*) within OFT cushions (Fig. S8). This differential gene expression change between AVC and OFT cushions suggests that myocardial β-catenin regulates the development of AVC and OFT cushions through different mechanisms.

### 3.3. Autocrine regulation of endocardial cushion growth by β-catenin in endocardium and mesenchyme

To determine whether β-catenin in endocardium and mesenchyme is required for cardiac cushion formation, we generated endocardial lineage-specific β-catenin knockout mice (β-cat$^{eKO}$, hereafter) by crossing floxed Ctnnb1 mice with the *Nfatc1$^{Cre}$* mice. Immunostaining showed that β-catenin protein in AVC endocardium of E9.5 β-cat$^{eKO}$ embryos was slightly decreased when compared to controls, while its expression in OFT endocardium of E9.5 β-cat$^{eKO}$ embryos was not affected at this stage (Fig. S9). At E10.5, β-catenin level in the endocardium and mesenchyme within AVC and proximal OFT regions was greatly diminished (Fig. S9). β-cat$^{eKO}$ embryos were lethal before E15.5 (Fig. S10A), but developed normally by E11.5, with gross morphology comparable to their littermate controls (*β-catenin$^{f/+}$* or *β-catenin$^{f/f}$*) (Fig. S10B). Of particular note, β-cat$^{eKO}$ embryos developed normal vasculature in the yolk sac at E10.5 (Fig. S10B), and are unlike the embryos with the pan-endothelial deletion of β-catenin that have vascular defects in the yolk sac [34]. β-cat$^{eKO}$ embryos began exhibiting a sign of underdevelopment at E12.5 and dying between E13.5 and E14.5 (Fig. S10B). These results demonstrate that β-catenin in the endocardium and mesenchyme is indispensible for embryo survival.

We then studied cardiac morphology by HE staining and found that E10.5 β-cat$^{eKO}$ embryos developed normal AVC and OFT cushions, evident by similar cushion area and number of mesenchymal cells to those of littermate controls (Fig. S10C and D, and Fig. S11). However, the subsequent development of AVC and OFT cushions in E11.5 and 12.5 β-cat$^{eKO}$ embryos was arrested (Fig. 4A, Fig. S12A, S13 and S14). EdU assays revealed significantly reduced

proliferation of endocardial and mesenchymal cells in AVC and OFT of E11.5 β-cat$^{eKO}$ embryos compared to controls (Fig. 4B and Fig. S12B). TUNEL labeling showed that endocardial deletion of β-catenin resulted in increased apoptosis of mesenchymal cells in AVC and OFT cushions of E11.5 embryos (Fig. 4C and Fig. S12C). In contrast, endocardial deletion of β-catenin had no impact on alcian blue staining (Fig. S15). At the molecular level, qPCR analysis showed that E11.5 β-cat$^{eKO}$ embryos had decreased mRNA levels of *Bmp6*, *Msx1* and *Id3* in their hearts (Fig. S16). Together, these results reveal a cell autonomous regulation of endocardial cushion growth by β-catenin in endocardium and mesenchyme during heart valve development.

### 3.4. WNT/β-catenin signaling is spatiotemporally activated in the heartvalve forming regions

β-catenin can form a transcriptional complex with TCF/LEF in nucleus and mediate canonical WNT signaling. The cushion defects resulting from tissue-specific inactivation of β-catenin in the endocardium, mesenchyme and myocardium led us to examine the canonical WNT activity in the heart valve-forming regions between E9.5 and E12.5. We traced WNT/β-catenin signaling using a transgenic reporter line specific for canonical WNT signaling in which GFP expression is under the control of 6 tandem TCF/LEF binding sites [35]. The results showed that a sustained high level of WNT/β-catenin activity was present in the AVC cushion myocardium from E9.5 to E12.5. In contrast, WNT/β-catenin activities in the endocardium and mesenchyme of the AVC cushion were undetectable or at a very low level at E9.5 and E10.5, and gradually increased after E10.5 (Fig. 5A and B). We found that canonical WNT/β-catenin activities in the OFT had a similar pattern of tissue distribution, although they appeared one day later compared to AVC (Fig. 5A and B). The spatiotemporal WNT/β-catenin activities suggest that β-catenin regulates endocardial cushion growth through its signaling function.

### 3.5. Signaling function of β-catenin critically regulates endocardial cushion growth

We therefore sought to determine whether the signaling function of β-catenin is required for cardiac cushion growth. To this end, we used a mouse model β-catenin$^{DM}$ that expresses a mutant β-catenin protein containing a single amino acid change (D164A) in the first armadillo repeat and a C-terminal truncation. Because these mutations prevent interacting with specific transcriptional coactivators, β-catenin$^{DM}$ lacks any detectable transcriptional activity but retains the intact cell adhesion function. By combining β-catenin$^{DM}$ with tissue-specific deletion of β-catenin, we were able to interrogate the role of the signaling function of β-catenin in endocardial cushion development. First, we crossed β-catenin$^{DM}$ and β-catenin$^{flox}$ mice with *Tnt*$^{Cre}$ mice to generate compound β-catenin mutant mice β-catenin$^{DM/flox}$:*Tnt*$^{Cre}$ (β-cat$^{DM/mKO}$, hereafter), while the mice with genotype β-catenin$^{DM/flox}$ or β-catenin$^{DM/+}$ were used as controls. Histology analysis showed that E11.5 β-cat$^{DM/mKO}$ embryos had hypoplastic AVC (Fig. 6A and C, and Fig. S17) and OFT (Fig. S18 and S19) cushions similar as that observed in β-cat$^{mKO}$ embryos, demonstrating that the signaling function of β-catenin in the cushion myocardium was essential for cushion growth. We next crossed β-catenin$^{DM}$ and β-catenin$^{flox}$ mice with *Nfatc1*$^{Cre}$ mice to generate

compound β-catenin mutant mice β-catenin$^{DM/flox}$:*Nfatc1*$^{Cre}$ (β-cat$^{DM/eKO}$, hereafter), while the mice with genotype β-catenin$^{DM/flox}$ or β-catenin$^{DM/+}$ were used as controls. Histology analysis showed that E11.5 β-cat$^{dm/eKO}$ embryos had hypoplastic AVC (Fig. 6B and D, and Fig. S20) and OFT (Fig. S17 and S21) cushions which were similar to the defects observed in β-cat$^{eKO}$ embryos, confirming that the signaling function of β-catenin in the cushion endocardium and mesenchyme was essential for cushion growth. Together, these findings indicate that β-catenin in myocardium, endocardium and mesenchyme regulates cushion growth through its signaling function.

## 4. Discussion

Based on the *in vitro* studies, we have previously proposed an endocardial-WNT to myocardial-BMP signaling axis underlying EMT process of endocardial cushion formation. Here we further investigated this model using a myocardial-specific β-catenin knockout mouse model and found the *in vivo* evidence to support that *Bmp2* is a mediator of WNT/β-catenin signaling in AVC cushion myocardium to promote mesenchymal cell proliferation and cushion expansion. In addition, we confirmed that WNT/β-catenin signaling in endocardium and mesenchyme positively regulates the growth of endocardial cushion growth. Our observations are summarized schematically in Fig. 7.

In this study, Tnt$^{Cre}$ was used to drive the deletion of β-catenin specifically in myocardium. This deletion caused embryonic lethality around E14.5 and had no effect on the gross embryo development before E11.5, evident by normal morphology and embryo size (Fig. S1E-G). In contrast, previous reports have shown that Islet1$^{Cre}$ or Nkx2.5$^{Cre}$ mediated deletion of β-catenin results in a more severe cardiac phenotype characterized by early embryonic lethality and delayed embryo development [36]. This difference between our and previous studies could be explained by the different Cre divers used in the studies. The Islet1$^{Cre}$ mice start to express Cre at E7.0-E7.5 in the mesodermal progenitors of second heart field that give rise to right ventricle and OFT [36]. The Nkx2.5$^{Cre}$ mice start to express Cre in cardiac crescent/straight heart tube around E7.5/8.0 of first heart field. Both Cres are thus expressed in cardiac progenitors giving rise to both cardiomyocytes and endocardial cells [36]. In contrast, Tnt$^{Cre}$ is known to be expressed specifically in cardiomyocytes [26]. Our immunostaining showed that significant deletion of β-Catenin drove by Tnt$^{Cre}$ started to be observed at E10.5 (Fig. S1B-D). Together, the later and cardiomyocyte-specific deletion may explain the milder myocardial phenotype caused by deletion of β-catenin using Tnt$^{Cre}$ than those caused by deletion of β-catenin using Islet1$^{Cre}$ or Nkx2.5$^{Cre}$.

BMP signaling is a well-known paracrine signal from myocardium for EMT and post-EMT mesenchymal cell proliferation during endocardial cushion formation [25,27,28,37,38]. Disruption of myocardial *Bmp2* inhibits EMT and results in hypocellular cushions [28,39], suggesting a critical role of *Bmp2* in EMT. Similarly, we have reported previously an endocardial to myocardial NOTCH-WNT-BMP signal axis for EMT during endocardial cushion formation [33]. Consistently, we show in this study that *Bmp2* expression in AVC cushions of E9.5 β-cat$^{mKO}$ embryos was decreased to 70% of that in controls. This slight but significant decrease is most likely due to incomplete deletion of β-catenin at this early

stage. Consistently, previous studies have reported that myocardial β-catenin is required for *Bmp2* expression and mitral valve formation [21]. In our study, early development of AVC in β-cat$^{mKO}$ embryos appears normal at E9.5 without changes in the expression of EMT markers (*Ve-cad, Notch1, Snail and slug*). However, cushion growth after EMT is inhibited in these embryos. These findings suggest that 70% of normal *Bmp2* level is sufficient for proper EMT, yet not for maintaining the high proliferation rate during post-EMT cushion growth. On the other hand, further reduction of BMP2 resulted from complete inactivation of β-catenin impairs EMT. In contrast to AVC cushions, the OFT cushions in β-cat$^{mKO}$ embryos showed unchanged or slightly increased expression of BMP/Tgfβ ligands including *Bmp2, Bmp4, Bmp5, Bmp6, Bmp7, Tgfβ1, Tgfβ2, and Tgfβ3*. These findings suggest that myocardial β-catenin regulates the growth of AVC and OFT cushions through different mechanisms.

Our genetic evidence strongly supports that *Bmp2* is a downstream target and mediator of WNT/β-catenin signaling in the AVC myocardium, although the mechanisms by which β-catenin controls the expression of *Bmp2* remains unknown. A positive feedback regulation between *Tbx2/3* and *Bmp2* has been reported to regulate AVC myocardial development [40], suggesting that *Tbx2/3* may work intermediately between WNT/β-catenin and *Bmp2* gene activation. The results of our gene expression analysis, however, indicate that *Bmp2* is decreased prior to the downregulation of *Tbx2/3* in E9.5 β-cat$^{mKO}$ embryos. Alternatively, *Msx1/2* has been shown to regulate the expression of *Bmp2* in AVC myocardium [41]. Our results also show that *Msx2* and *Bmp2* are decreased in β-cat$^{mKO}$ embryos simultaneously (Fig. S7A). Based on these observations, we speculate that WNT/β-catenin signaling may regulate *Bmp2* through *Msx2*. Nonetheless, further investigation is needed to test whether *Bmp2* is a direct target of β-catenin in the AVC myocardium as previously suggested in osteoblasts and embryonic stem cells [42,43].

Previous studies by deleting β-catenin in pan-endothelium with *Tie2$^{Cre}$* indicate an essential role of β-catenin in EMT [22]. However, these β-catenin mutant embryos die before E10.5 because of multiple defects in heart as well as yolk sac vessels [34]. In our study, we deleted β-catenin specifically in endocardium and its mesenchymal progeny using the *Nfatc1$^{cre}$* driver line. Unlike pan-endothelial β-catenin knockouts, mice with endocardial deletion of β-catenin develop normal yolk sac vasculature and survive till to E15.5, allowing us to study the role of β-catenin in post-EMT cushion growth. Our tissue-specific deletion shows definitively that endocardial β-catenin is required for endocardial cushion formation through promoting cell proliferation. This finding is consistent with the previous report that inhibition of WNT/β-catenin signaling by overexpressing DKK1 inhibits cushion growth after EMT [20].

TOPGAL, BATGAL and *Axin2$^{LacZ}$* reporter mouse lines are widely used during heart development with different findings. BATGAL mice show that WNT/β-catenin signaling is predominantly present in the endocardium and the cushion mesenchyme at E10.5, suggesting a role for WNT/β-catenin signaling in promoting EMT [22]. In contrast, *Axin2$^{LacZ}$* mice show that WNT/β-catenin signaling is primarily located in the cushion myocardium and is rare in the endocardium at E9.5 and E10.5, which argues against a

direct role for WNT/β-catenin signaling in EMT [20]. Our study uses a new reporter mouse line to examine the WNT/β-catenin signaling in the heart valve-forming region. This line expresses an H2B-EGFP fusion protein under the control of six copies of a T-cell specific transcription factor/lymphoid enhancer-binding factor 1 (TCF/Lef1) response element and a heat shock protein 1B (*Hspa1b*) minimal promoter [35], thus directly reporting the nuclear transcriptional activation by β-catenin. We show here that WNT/β-catenin signaling is dominant in cushion myocardium and rare in endocardium at E9.5, suggesting that EMT is unlikely dependent on WNT/β-catenin signaling in endocardium. This is consistent with the previous report that inhibition of WNT/β-catenin signaling by overexpressing DKK1 has no effect on EMT within the AVC cushion [20]. After E10.5, WNT/β-catenin activity is retained at a high level in cushion myocardium and begins to increase in cushion mesenchyme, and together they execute paracrine and autocrine functions necessary for post-EMT cushion growth, as shown by using a strategy of compound deletion of a floxed allele β-catenin$^{flox}$ and a mutant allele β-catenin$^{DM}$ that inactivates the β-catenin signaling function, but retains its cell-cell adhesion function.

The canonical WNT signaling is present in a small cell population of endocardium and mesenchyme during endocardial cushion growth and their loss likely will not cause a dramatic cushion defect. However, the similar cushion defects observed in β-cat$^{eKO}$ and β-cat$^{DM/eKO}$ suggest that the signaling function of β-catenin in endocardium and mesenchyme is also essential for cushion development. β-catenin interacts with many proteins, such as TCF/LEF, B-cell lymphoma9 (BCL9), TATA-binding protein (TBP), Brahma/Brahma-relatedgene-1 (Brg-1), CREB-binding protein (CBP)/p300, Mediatorsubunit 12 (MED12), and Hyrax/Parafibromin [44]. Disruption of these interactions in β-cat$^{dm}$ allele likely blocks the canonical WNT signaling, as well as other signals. Together, their dysregulation may contribute to the cushion phenotypes.

In conclusion, our findings demonstrated that WNT/β-catenin signaling in myocardium promotes mesenchymal cell proliferation and cushion expansion through inducing *Bmp2* expression in myocardium and the resulting BMP/SMAD signaling in mesenchyme. This new information would advance our current understanding of the mechanisms underlying endocardial cushion formation and congenial heart valve disease.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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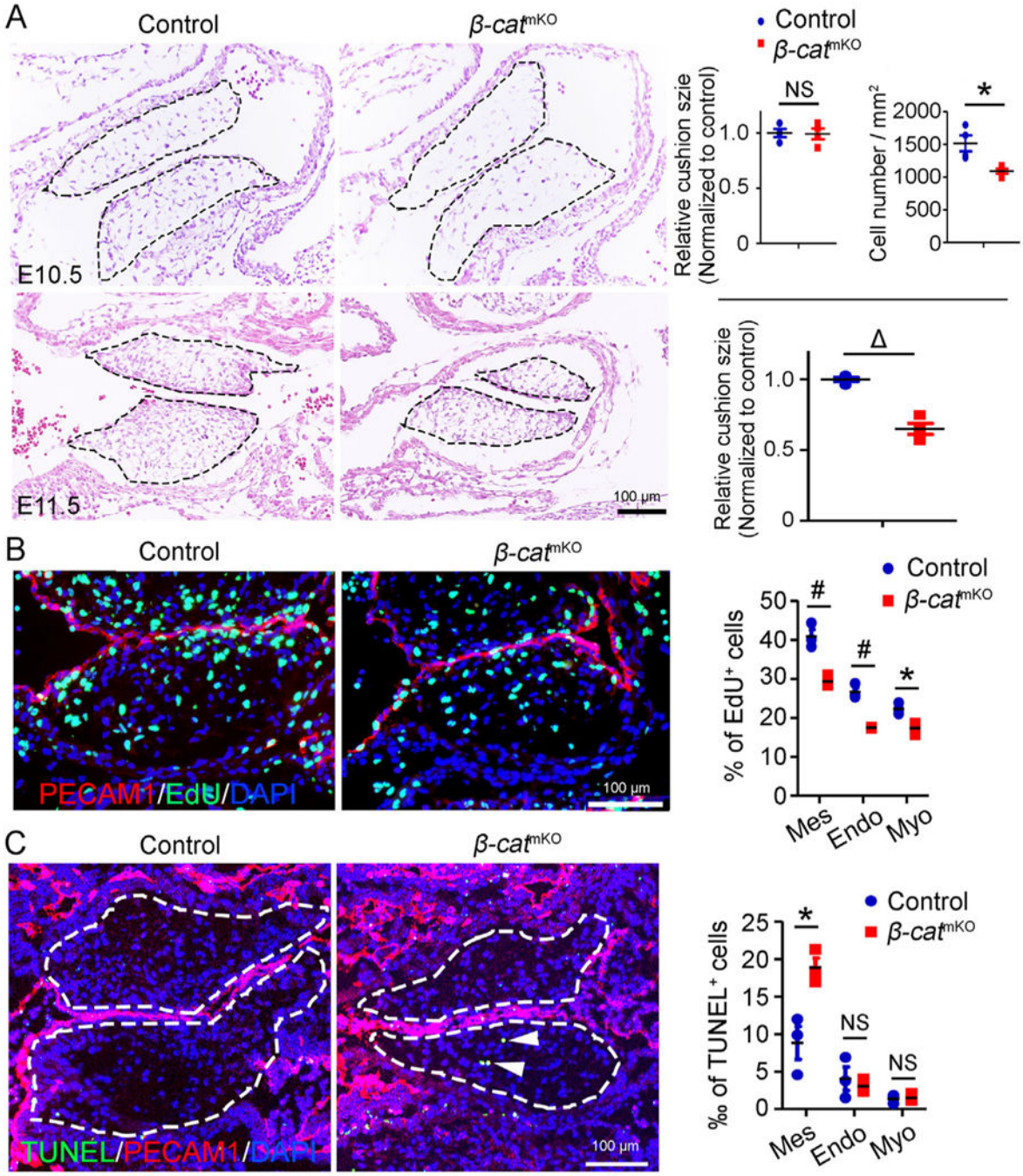


**Fig. 1.**

Paracrine regulation of AVC cushion growth by myocardial β-catenin.

(A) Representative images from HE stained tissue sections from E10.5 and E11.5 control (*β-cat*$^{f/f}$ or *β-cat*$^{f/+}$) and myocardial-specific *β-catenin* knockout (*β-cat*$^{f/f}$:*Tnt*$^{Cre}$, β-cat$^{mKO}$) embryos show atrioventricular canal (AVC) cushions (outlined by dashed line). The bar charts show the quantification of cushion area and cell density. *n* = 4/group. (B) Representative images of EdU assays show the proliferating cells (green) in E11.5 AVC cushions. The cushion endothelial cells were stained with PECAM1 (red). The bar chart

shows the quantification of cell proliferating rate in mesenchyme (Mes), endocardium (Endo) or myocardium (Myo) of AVC cushions. $n$ = 3/group. (C) Representative images of TUNEL analysis show the apoptotic cells (green) in AVC cushion region. The cushion endothelial cells were stained with PECAM1 (red). The bar chart shows the quantification of cell apoptotic rate in mesenchyme, endocardium or myocardium of AVC cushions. n = 3/group. Statistical calculation was performed using unpaired two-way student *t*-test. * < 0.05. # < 0.01. Δ < 0.001. NS, No significance. The scale bars represent 100 μm.

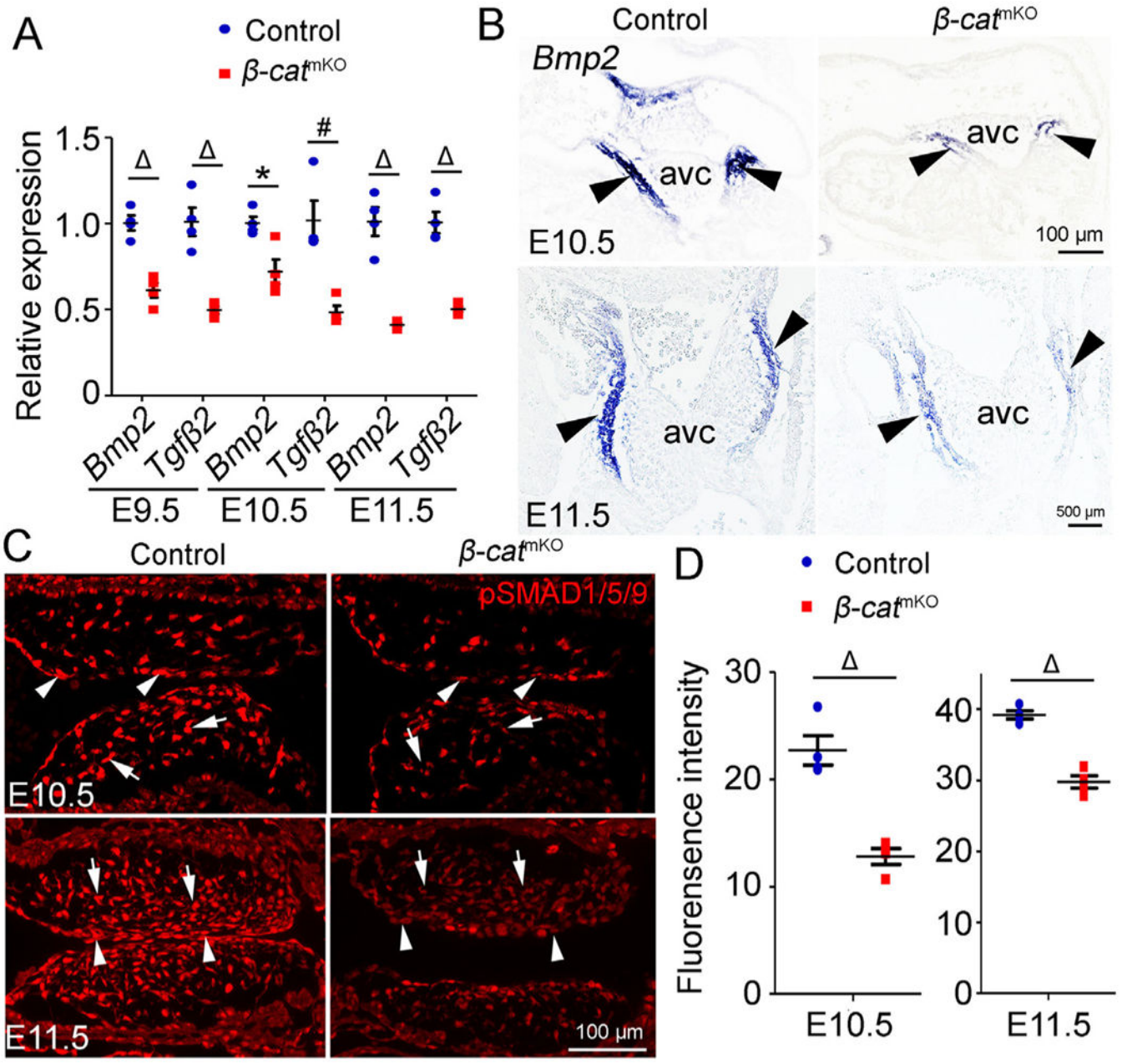


**Fig. 2.**

Loos of myocardial β-catenin disrupts *Bmp2* expression in myocardium and the BMP/SMAD signaling in mesenchyme.

(A) Quantitative PCR (qPCR) analysis of the expression of *Bmp2* and *Tgfb2* genes in AVC cushion tissues from E9.5, E10.5 and E11.5 control (β-cat$^{f/f}$ or β-cat$^{f/+}$) and β-cat$^{mKO}$ (β-cat$^{f/f}$:Tnt$^{Cre}$) embryos. The expression of *Bmp2* and *Tgfb2* was normalized to that of *Gapdh*. Tissues from two to four embryos were pooled as one sample. n = 4/group.

(B) Representative images of *RNA in situ* hybridization analysis show *Bmp2* expression in myocardium of AVC cushion (arrowheads) of E10.5 and E11.5 control and β-cat$^{mKO}$ embryos. n = 3–5/group. (C and D) immunofluorescence shows the expression of phospho-

SMAD1/5/9 (p-SMAD1/5/9) (red) in AVC cushions of E10.5 and E11.5 control and β-cat$^{mKO}$ embryos. Arrows and arrowheads indicate pSMAD1/5/9 positive mesenchymal and endocardial cells, respectively. The bar charts show the quantification of the mean fluorescence intensity of pSMAD1/5/9 staining. n = 4/group. Statistical calculation was performed using unpaired two-way student *t*-test. * < 0.05. # < 0.01. Δ < 0.001.

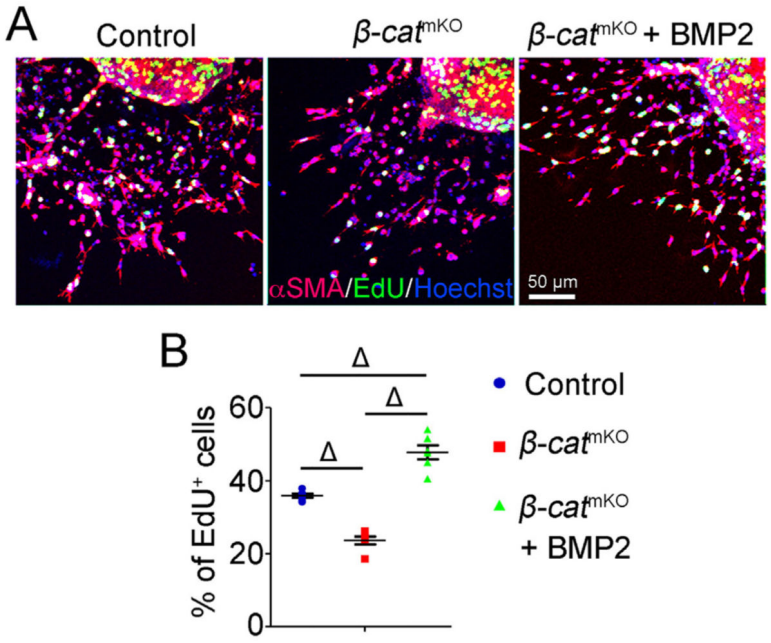


**Fig. 3.**

BMP2 mediates myocardial β-catenin to promote mesenchymal cell proliferation.

(A and B) AVC cushions from E10.5 control (*β-cat*$^{f/f}$ or *β-cat*$^{f/+}$) and β-cat$^{mKO}$ (*β-cat*$^{f/f}$:*Tnt*$^{Cre}$) embryos were cultured on collagen gels with or without BMP2 (200 ng/ml) for 48 h. Representative images from EdU analysis of these explants show proliferating cells (green). Mesenchymal cells were highlighted by immunostaining of αSMA (red), while cell nuclei were stained with Hoechst (blue). The bar chart shows the quantification of cell proliferating rate in indicated conditions. $n$ = 5/group. Statistical calculation was performed using unpaired two-way student $t$-test. Δ < 0.001.

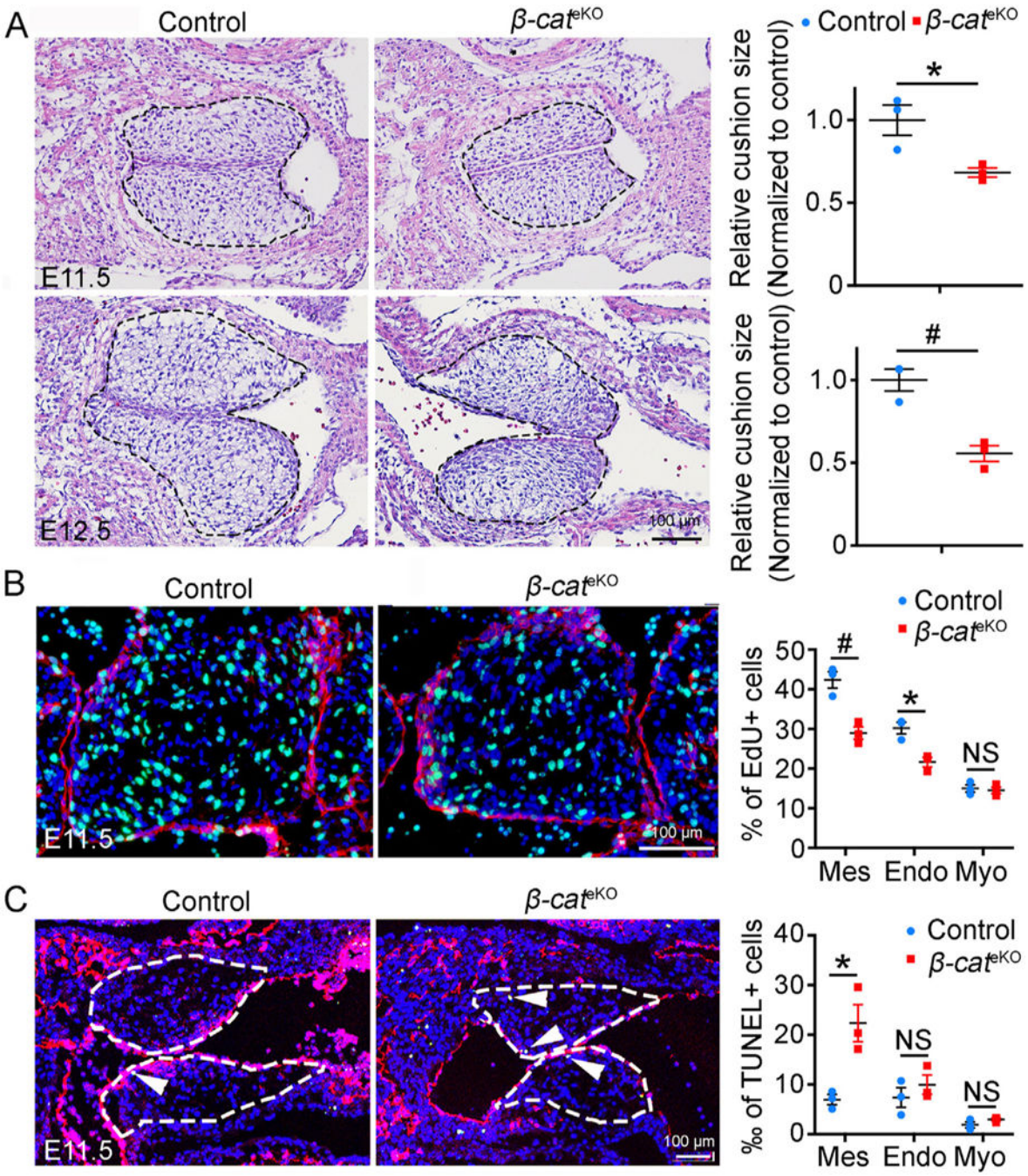


**Fig. 4.**

Autocrine regulation of AVC cushion growth by β-catenin in the endocardium and mesenchyme.

(A) Representative images from HE stained tissue sections of E11.5 and E12.5 control ($\beta$-*cat*$^{f/f}$ or $\beta$-*cat*$^{f/+}$) and endocardial-specific $\beta$-*catenin* knockout ($\beta$-*cat*$^{f/f}$:*Nfatc1*$^{Cre}$, $\beta$-*cat*$^{eKO}$) embryos show the regions of atrioventricular canal (AVC) cushions (outlined by dashed line). The bar charts show the quantification of cushion area. n = 3/group. (B) Representative images of EdU assay show the proliferating cells (green) in E11.5 AVC

cushions. The cushion endothelial cells were stained with PECAM1 (red). The bar chart shows the quantification of cell proliferating rate in mesenchyme (Mes), endocardium (Endo) or myocardium (Myo) of AVC cushions. n = 3/group. (C) Representative images of TUNEL analysis show the apoptotic cells (green) in AVC cushion region. The cushion endothelial cells were stained with PECAM1 (red). The bar chart shows the quantification of cell apoptotic rate in mesenchyme, endocardium or myocardium of AVC cushions. n = 3/group, Statistical calculation was performed using unpaired two-way student *t*-test. * < 0.05. # < 0.01. NS, No significance.

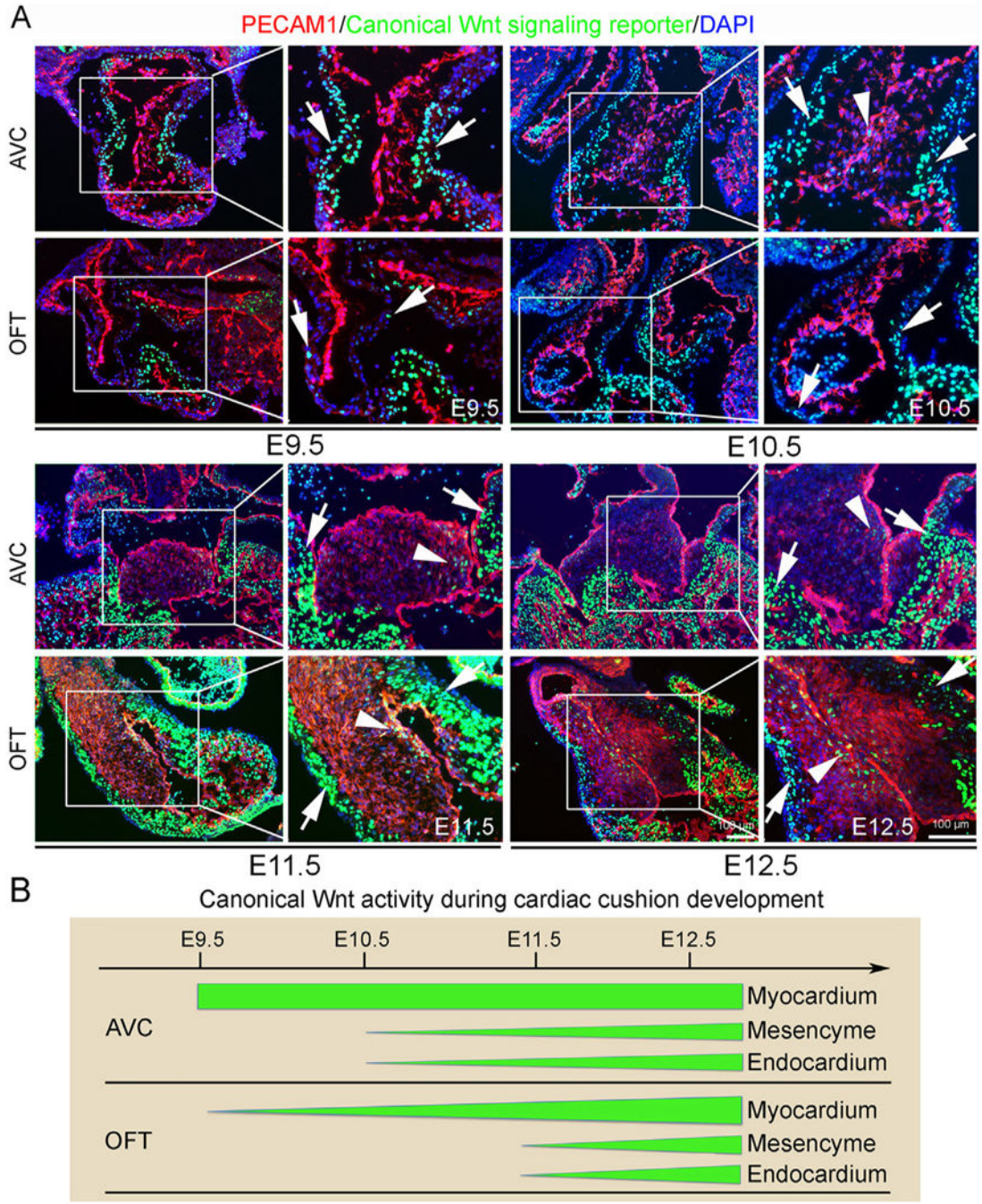


**Fig. 5.**

Spatiotemporal Wnt/β-catenin signaling in cushion-forming region.

(A) Representative images of the heart valve-forming region at AVC or OFT from E9.5 to E12.5 hearts of the transgenic Wnt/β-catenin signaling reporter embryos show Wnt/β-catenin signaling (GFP) in the cushion myocardium (indicated by arrows), endocardium or mesenchyme (indicated by arrowheads). PECAM1 staining marks the endocardium (red). (B) Schematic summary of Wnt/β-catenin signaling in myocardium, mesenchyme and endocardium of AVC and OFT cushions from E9.5 to E12.5. At AVC, high levels of Wnt/

β-catenin signaling are present in the myocardium at all stages, whereas the mesenchyme or endocardium begins to express low levels of Wnt/β-catenin signaling at E10.5, which gradually increases at later stages. Wnt/β-catenin signaling is present in OFT myocardium at a low level at E9.5 and gradually increases at later stages. Low levels of Wnt/β-catenin signaling in the OFT mesenchyme or endocardium are observed after E10.5.

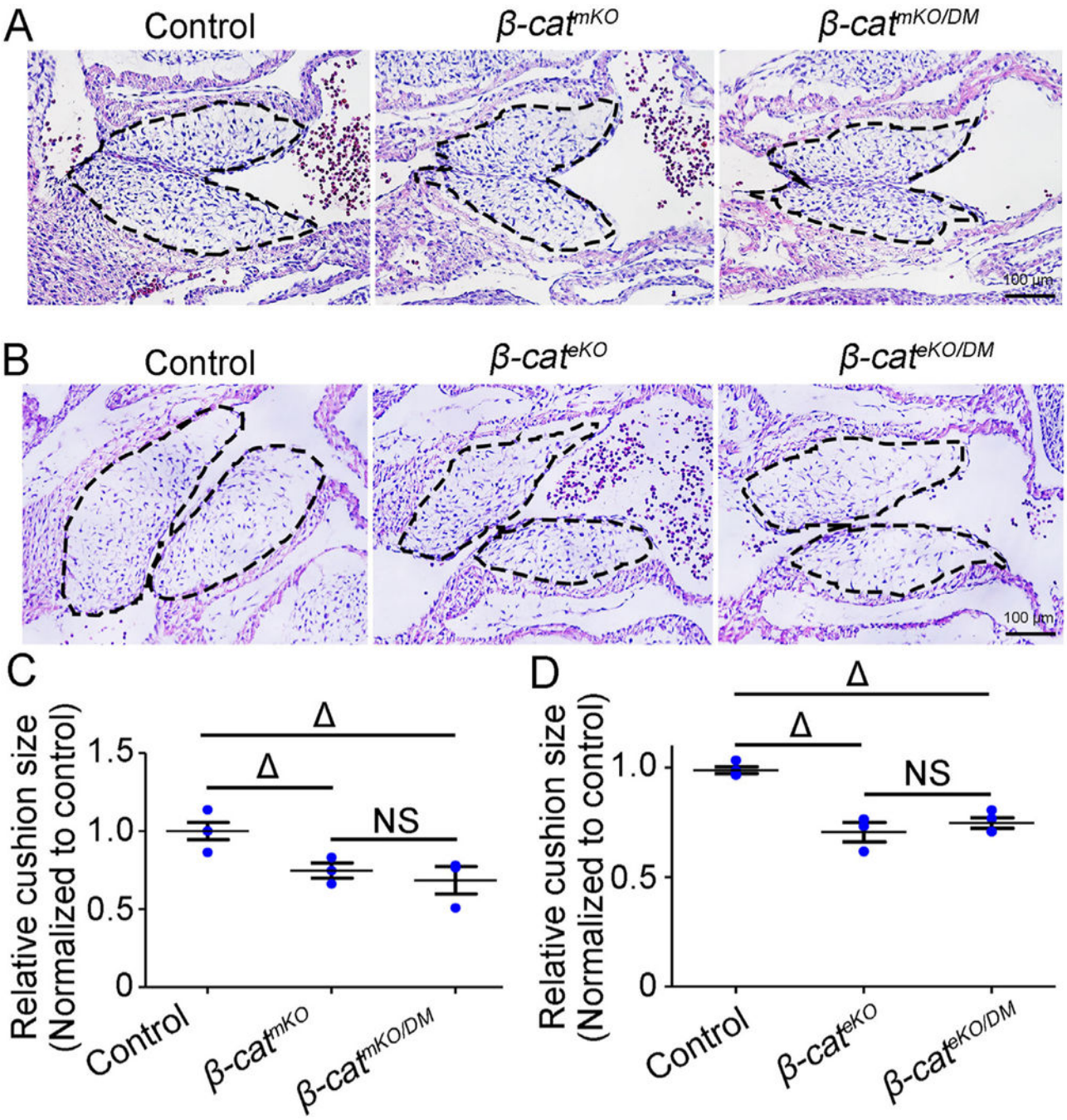


**Fig. 6.**

Signaling function of β-catenin is essential for endocardial cushion growth.

(A) representative images from HE stained tissue sections show atrioventricular canal (AVC) cushions (outlined by dashed line) in E11.5 control (*β-cat*$^{f/DM}$ or *β-cat*$^{DM/+}$), β-cat$^{mKO}$ (*β-cat*$^{f/f}$:*Tnt*$^{Cre}$), and β-cat$^{mKO/DM}$ (*β-cat*$^{f/DM}$:*Tnt*$^{Cre}$) embryos. (B) representative images from HE stained tissue sections show AVC cushions (outlined by dashed line) in E11.5 control (*β-cat*$^{f/DM}$ or *β-cat*$^{DM/+}$), β-cat$^{eKO}$ (*β-cat*$^{f/f}$:*Nfatc1*$^{Cre}$), and β-cat$^{eKO/DM}$ (*β-cat*$^{f/DM}$:*Nfatc1*$^{Cre}$) embryos. (C and D) The bar charts show the quantification of cushion

size showed in panel A (C) and panel B (D). The cushion size was normalized to controls. n = 3/group. $\Delta < 0.001$. NS, No significance.

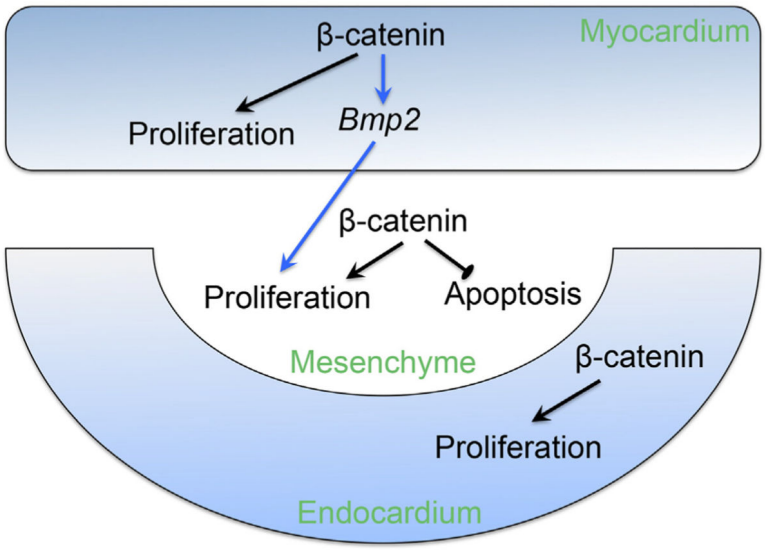


**Fig. 7.**

Schematic summaries of autocrine and paracrine regulation of endocardial cushion formation by β-catenin.

The schematic model summaries tissue specific roles of β-catenin during AVC cushion formation. Black arrows indicate the autocrine regulation while blue arrows indicate paracrine regulation. Myocardial β-catenin regulates the proliferation of mesenchyme in a paracrine manner through inducing expression of *Bmp2*. Meanwhile, β-catenin in endocardium and mesenchyme promotes cell proliferation cell autonomously.